Coupling of low-molecular substances to a modified polysaccharide

There is a large number of low molecular weight substances of commercial interest, especially active pharmaceutical ingredients and crop protection agents, whose use is limited or even prevented by unsatisfactory solubility properties in an aqueous medium and/or short residence time in the body. Thus, for example, small pharmaceutical molecules are frequently removed from the circulation again too quickly by glomerular filtration in the kidney (exclusion limit about 70 kD), so that continual replenishment, which is costly and inconvenient for the patient, with this medicament is necessary, e.g. by frequently repeated administrations or infusion.

In order to avoid this disadvantage, in some cases slightly soluble active pharmaceutical ingredients are administered as an oily bolus which frequently forms painful deposits at the injection site. In addition, the use of such slightly soluble medicaments is often associated with toxic side effects because of their deposition in organs such as liver and/or kidney. Such unwanted side effects in turn result in the concentration range which can be employed in vivo for the active ingredient being greatly restricted.

An approach followed in recent times for eliminating the described problems consists of coupling such problematic substances to readily soluble biocompatible polymers such as, for example, polyethylene glycol and dextran. It is possible through the coupling on the one hand to increase the molecular weight above the threshold of 70 kD, so that the plasma residence time of smaller molecules can be drastically increased, and on the other hand the solubility in aqueous medium can be improved by the hydrophilic polymer portion.

Most modifications to date have been carried out with polyethylene glycol or dextran, with PEG being generally preferred because it yields simpler products. Dextran conjugates often show high allergenicity, a low metabolic stability and, in many cases, low yields of the coupling reactions. There have likewise been reports of unpleasant or hazardous side effects such as pruritus, hypersensitivity reactions and pancreatitis on use of PEG conjugates. In addition, the biological activity of the active ingredients is more often greatly reduced in some cases after the PEG coupling. Moreover, the metabolism of the degradation products of PEG conjugates is still substantially unknown and possibly represents a health risk.

Thus, there is still a need for physiologically well tolerated alternatives to dextran or PEG conjugates, with which the solubility of poorly soluble low molecular weight substances can be improved and/or the residence time of low molecular weight substances in the plasma can be increased, resulting in improved pharmacodynamic properties of the active molecule.

It is therefore an object of the invention to provide such alternatives and to develop simple and efficient methods for preparing such alternative conjugates.

It has surprisingly been found that this object can be achieved by hydroxyalkylstarch conjugates which are characterized in that the binding interaction between the hydroxyalkylstarch molecule and the low molecular weight substance is based on a covalent bonding which is the result of a coupling reaction between the terminal aldehyde group, or a functional group derived from this aldehyde group by chemical reaction, of the hydroxyalkylstarch molecule and a functional group, which is able to react with this aldehyde group or functional group derived therefrom of the

hydroxyalkylstarch molecule, of the low molecular weight substance, where the bonding resulting directly in the coupling reaction can be modified where appropriate by a further reaction to give the abovementioned covalent bonding.

The invention further includes pharmaceutical compositions which comprise these conjugates, and the use of these conjugates and compositions for the prophylactic or therapeutic treatment of the human or animal body, and methods for preparing these conjugates and compositions.

The hydroxyalkylstarch (HAS) employed according to the invention can be prepared by a known method, e.g. hydroxyalkylation of starch at the C_2 and/or C_6 position of the anhydroglucose units with alkylene oxide or 2-chloroalkanol, e.g. 2-chloroethanol (see, for example, US 5 218 108 for the hydroxyethylation of starch), with various desired molecular weight ranges and degrees of substitution. It is also possible to employ any preparations obtainable commercially. The definition of the alkyl grouping in "hydroxyalkylstarch", as used herein, includes methyl, ethyl, isopropyl and n-propyl, with particular preference for ethyl. A substantial advantage of hydroxyethylstarch (HES) is that it is already approved by the authorities as biocompatible plasma expander and is employed clinically on a large scale.

The average molecular weight of the hydroxyalkylstarch can be in the range from about 3 kD to several million daltons, preferably about 10 kD to about 200 kD, more preferably in the range from about 70 kD to about 1000 kD, particularly preferably about 130 kD. To increase the residence time of the low molecular weight substance in the organism, the average molecular weight of the hydroxyalkylstarch is preferably chosen so that the glomerular threshold of 70 kD is exceeded with the

conjugates. The degree of substitution (ratio of the number of modified anhydroglucose units to the number of anhydroglucose units in total) may likewise vary and will frequently be in the range from about 0.2 to 0.8, preferably about 0.3 to 0.7, more preferably about 0.5. (Note: the numbers relate to the "degree of substitution", which is between 0 and 1). The ratio of C_2 to C_6 substitution is normally in the range from 4 to 16, preferably in the range from 8 to 12.

These parameters can be adjusted by known methods. Experience with the use of hydroxyethylstarch as blood substitute has shown that the residence time of HES in the plasma depends on the molecular weight and the degree of substitution and type of substitution (C_2 substitution or C_6 substitution), with a higher molecular weight, a higher degree of substitution and a higher proportion of C_2 substitution increasing the residence time.

These relationships also apply to the inventive conjugates of hydroxyalkylstarch and low molecular weight substances, so that the residence time of a particular conjugate in the plasma can be adjusted via the proportion of polysaccharide.

As already mentioned, the functional group involved in the coupling reaction of the hydroxyalkylstarch molecule is the terminal aldehyde group or a functionality derived therefrom by chemical reaction.

One example of such a chemical reaction is the selective oxidation of this aldehyde group with a suitable oxidizing agent such as, for example, iodine, bromine or some metal ions, or else by means of electrochemical oxidation to a carboxyl group or activated carboxyl group, e.g. an ester, lactone, amide, with the carboxyl group being converted where appropriate in a second reaction into the activated

derivative. This carboxyl group or activated carboxyl group can then be coupled to a primary amino or thiol group of the low molecular weight substance to form an amide linkage or thioester linkage. A further possibility is coupling to a hydroxyl function of the low molecular weight substance to form an ester.

An inventive conjugate can, however, also be obtained by reacting the low molecular weight substance with a suitable physiologically tolerated bifunctional linker molecule to introduce a desired functional group. The remaining reactive group of the coupled-on linker molecule is likewise for the purposes of the present invention considered to be a "reactive functional group of the low molecular weight substance".

Suitable linker molecules comprise at one end a grouping able to enter into a covalent bonding with a reactive functional group of the low molecular weight substance, e.g. an amino, thiol, carboxyl or hydroxy group, and at the other end a grouping likewise able to enter into a covalent bonding with the terminal aldehyde group or a functional group derived therefrom by chemical reaction, e.g. a carboxyl group, activated carboxyl group, amino or thiol group.

Between the two functional groups of the linker molecule there is a biocompatible bridging molecule of suitable length, e.g. a grouping derived from an alkane, an (oligo)alkylene glycol grouping or another suitable oligomer grouping. Preferred groupings able to react with amino groups are, for example, N-hydroxysuccinimide esters, sulfo-N-hydroxysuccinimide esters, imido esters or other activated carboxyl groups; preferred groupings able to react with thiol groups are, for example, maleimide and carboxyl groups; preferred groupings able to react with aldehyde or carboxyl groups are, for example, amino or thiol groups.

Examples of linker molecules for connecting SH and NH functions are:

AMAS	(N-α(maleimidoacetoxy)succinimide ester)
BMPS	(N-β(maleimidopropyloxy)succinimide ester)
GMBS	(N-γ(maleimidobutyryloxy)succinimide ester)
EMCS	(N-ε(maleimidocaproyloxy)succinimide ester)
MBS	(m-(maleimidobenzoyl)-N-hydroxysuccinimide ester)
SMCC	(succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate)
SMPB	(succinimidyl 4-(p-maleimidophenyl)butyrate)
SPDP	(succinimidyl 3-(2-pyridyldithio)proprionate)
Sulfo-GMBS	(N-γ(maleimidobutyryloxy)sulfosuccinimide ester)
Sulfo-EMCS	(N-ε(maleimidocaproyloxy)sulfosuccinimide ester).

Examples of linker molecules for connecting SH and SH functions are:

BMB	(1.4-bis-maleimidobutane)
BMDB	(1.4-bis-maleimido-2,3-dihydroxybutane)
BMH	(bis-maleimidohexane)
BMOE	(bis-maleimidoethane)
DTME	(dithio-bis-maleimidoethane)
HBVS	(1.6-hexane-bis-vinyl sulfone)
$BM(PEO)_3$	(1.8-bis-maleimidotriethylene glycol)
$BM(PEO)_4$	(1.11-bis-maleimidotetraethylene glycol).

Examples of linker molecules for connecting NH and NH functions are:

BSOCOES	(bis-(2-succinimidyloxycarbonyloxy)ethyl) sulfone
BS^3	(bis-(sulfosuccinimidyl) suberate)
DFDNB	(1.5-difluoro-2,4-nitrobenzene)
DMA	(dimethyl adipimidate HCl))
DSG	(disuccinimidyl glutarate)
DSS	(disuccinimidyl suberate)
EGS	(ethylene glycol bis(succinimidyl succinate).

Examples of linker molecules for connecting SH and CHO functions are:

BMPH	(N-(β-maleimidopropionic acid)hydrazide TFA)
EMCA	(N-(ε-maleimidocaproic acid)hydrazide)
KMUH	(N-(κ-maleimidoundecanoic acid)hydrazide)
M_2C_2H	(4-(N-maleimidomethyl)cyclohexane-1-carboxyl-hydrazide HCl)
MPBH	(4-(4-N-maleimidophenyl)butyric acid hydrazide HCl)
PDPH	(3-(2-pyridyldithio)propionylhydrazide).

An example of a linker molecule for connecting SH and OH functions is
PMPI (N-(p-maleimidophenyl) isocyanate).

Examples of linker molecules for converting an SH function into a COOH function are

BMPA	(N-β-maleimidopropionic acid)
EMCH	(N-β-maleimidocaproic acid)
KMUA	(N-κ-maleimidoundecanoic acid).

Examples of linker molecules for converting an NH function into a COOH function are MSA (methyl N-succinimidyl adipate) or longer-chain homologues thereof or corresponding derivatives of ethylene glycol.

Examples of linker molecules for converting a COOH function into an NH function are DAB (1.4-diaminobutane) or longer-chain homologues thereof or corresponding derivatives of ethylene glycol.

An example of a linker molecule which reacts with an amino group of a molecule and provides a protected amino group at a larger distance from this molecule to avoid steric hindrance is TFCS (N-ε(trifluoro-acetylcaproyloxy)succinimide ester).

Further suitable linker molecules are known to skilled workers and commercially available or can be designed as required and depending on the functional groups present and desired in the HAS and the lower molecular weight substances to be coupled on, and be prepared by known methods.

In a particularly preferred preparation method, the terminal aldehyde group of HAS is selectively oxidized with a molar excess of iodine, preferably in a molar ratio of iodine to HAS of from 2:1 to 20:1, particularly preferably about 5:1 to 6:1, in aqueous basic solution. In the optimized method described in example 1, initially an amount of hydroxyalkylstarch is dissolved in hot distilled water, and somewhat less than 1 mole equivalent of aqueous iodine solution, preferably in a concentration of about 0.05-0.5N, particularly preferably about 0.1N, is added. After this, an aqueous NaOH solution in a molar concentration which is about 5-15 times, preferably about 10 times, that of the iodine solution is slowly added dropwise, at intervals of several minutes, to the reaction solution until the solution starts to become clear again after the addition. Somewhat less than 1 mole equivalent of the above aqueous iodine solution is again added to the reaction solution, the dropwise addition of the NaOH solution is resumed, and the addition of iodine and NaOH are repeated until an approximately 5.5-6 mole-equivalent iodine solution and an 11-12 mole-equivalent NaOH solution, based on the hydroxyalkylstarch, have been added. The reaction is then stopped, the reaction solution is desalted, e.g. by dialysis or ultrafiltration, subjected to a cation exchange chromatography, and the reaction product is obtained by lyophilization. In this method, virtually quantitative yields are achieved irrespective of the molecular weight of the HAS.

In a further particularly preferred embodiment, the

selective oxidation takes place with alkaline stabilized solutions of metal ions, e.g. Cu^{++} or Ag^{+}, likewise in approximately quantitative yields (Example 2). It is preferred in this case to employ an approximately 3-10 times molar excess of the oxidizing agent.

The selectively oxidized hydroxyalkylstarch which has formed is subsequently reacted in a suitable organic solvent with a primary amino group of the desired low molecular weight substance to form an amide linkage. Preferred solvents have been selected from the group of polar nonprotic solvents, and dimethyl sulfoxide (DMSO) has been particularly preferably used. In contrast to conventional methods described in the literature for similar coupling reactions, in this case it has surprisingly been found that the use of otherwise obligatory activators such as carbodiimides and triazoles is unnecessary. The coupling of selectively oxidized hydroxyethylstarch (ox-HES) to various model compounds (see examples) proceeded smoothly even in the absence of an activator.

However, the coupling reactions preferably take place in the presence of a carbodiimide, more preferably in the presence of DCC (dicyclohexyldicarbodiimide), most preferably in the presence of EDC (1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide).

The reactive group of the hydroxyalkylstarch molecule can also be an amine or thiol group produced by chemical reaction of the terminal aldehyde group. For example, a reductive amination of the aldehyde group can be carried out by reaction with ammonia in the presence of hydrogen and a catalyst or in the presence of sodium cyanoborohydride. The resulting amine or thiol group can then react with a free carboxyl group or aldehyde group of the low molecular weight substance. The initial results in this case are amide

or thioester linkages or Schiff's bases, which can be modified where appropriate by a further reaction.

A further possibility is for the terminal aldehyde group of the hydroxyalkylstarch molecule or a functional group derived therefrom by chemical reaction also to be reacted with a suitable physiologically tolerated bifunctional linker molecule. In this case, the "functional group derived from the terminal aldehyde group of the hydroxyalkylstarch molecule by chemical reaction" for the coupling reaction is the remaining reactive functional group of the bifunctional linker molecule with which the terminal aldehyde group or the functional group derived therefrom has been reacted. It is possible in this way likewise to convert the terminal aldehyde group into a desired functional group.

Suitable linker molecules comprise at one end a group able to enter into a covalent bonding with the terminal aldehyde group or a functional group derived therefrom by chemical reaction, e.g. a carboxyl group, activated carboxyl group, amino or thiol group, and at the other end a group being able to enter into a covalent bonding with a reactive functional group of the low molecular weight substance, e.g. an amino, thiol, carboxyl or OH group, preferably aryl-OH group. Between the two functional groups of the linker molecule there is a biocompatible bridging molecule of suitable length, e.g. a grouping derived from an alkane, an (oligo)alkylene glycol grouping or another suitable oligomer grouping. Preferred groupings able to react with amino groups are, for example, N-hydroxysuccinimide esters, sulfo-N-hydroxysuccinimide esters, imido esters or other activated carboxyl groups; preferred groupings able to react with thiol groups are, for example, maleimide and carboxyl groups; preferred groupings able to react with aldehyde or carboxyl groups are, for example, amino or thiol

groups.

A number of specific, non-restrictive examples of suitable linker molecules have already been indicated above with reference to the conjugation of linker molecules to low molecular weight substances.

In an alternative inventive coupling method of the present invention, the terminal aldehyde group of the hydroxyalkylstarch (HAS) is reacted directly with a primary amino group of the low molecular weight substance or of a linker molecule coupled to this substance, to form a Schiff's base. The formed Schiff's base is, subsequent or parallel thereto, reduced to the amine by reaction with a suitable reducing agent, resulting in a bonding which is stable in aqueous medium between low molecular weight substance and HAS.

Preferred reducing agents are sodium borohydride, sodium cyanoborohydride, organic boron complexes, e.g. a 4-(dimethylamino)pyridine-boron complex, N-ethyl-diisopropylamine-boron complex, N-ethylmorpholine-boron complex, N-methylmorpholine-boron complex, N-phenyl-morpholine-boron complex, lutidine-boron complex, triethylamine-boron complex, trimethylamine-boron complex; suitable stereoselective reducing agents are, for example, sodium triacetate borohydride, sodium triethylborohydride, sodium trimethoxyborohydride, potassium tri-sec-butylborohydride (K-Selectride), sodium tri-sec-butylborohydride (N-Selectride), lithium tri-sec-butylborohydride (L-Selectride), potassium triamylborohydride (KS-Selectride) and lithium triamyl-borohydride (LS-selectride).

The coupling reaction of HAS or oxidized HAS to a low molecular weight substance is, because the solubility in water of the substance is expected to be poor and the stability of the lactone in aqueous medium is low, preferably carried out in an organic solvent, more

preferably in a polar, nonprotic solvent in which the HAS and preferably also the low molecular weight substance is soluble. Examples of suitable solvents for HAS are DMSO, glycol, diglycol, triglycol and N-methylpyrrolidone. It is also possible to employ mixtures of DMSO with other solvents if the low molecular weight substance is insoluble in DMSO or another preferred solvent for HAS. The reaction can, however, also sometimes be carried out advantageously in heterogeneous phase.

The molar ratio of HAS to low molecular weight substance in the coupling reaction is usually about 20:1 to 1:1, preferably about 5:1 to 1:1.

The coupling yields based on the low molecular weight substance are usually more than 40%, frequently more than 60% and not uncommonly more than 80% (cf. examples).

The low molecular weight substance to be coupled is preferably an active pharmaceutical ingredient whose solubility in aqueous medium and/or whose bioavailability, stability and residence time in the body are to be increased. The term "low molecular weight substance" is intended also to include peptides of up to about 50 amino acids. The active pharmaceutical ingredient is preferably selected from the group composed of antibiotics, antidepressants, antidiabetics, antidiuretics, anticholinergics, antiarrhythmics, antiemetics, antitussives, antiepileptics, antihistamines, antimycotics, antisympathotonics, antithrombotics, androgens, antiandrogens, estrogens, antiestrogens, antiosteoporotics, antitumor agents, vasodilators, other antihypertensive agents, antipyretic agents, analgesics, antiinflammatory agents, β-blockers, immunosuppressants and vitamins.

Some non-restrictive examples of active pharmaceutical

ingredients having an NH_2 group as partner in the coupling reaction with HAS are:
albuterol, alendronate, amikazin, ampicillin, amoxicillin, amphotericin B, atenolol, azathioprine, cefaclor, cefadroxil, cefotaxime, ceftazidime, ceftriaxone, cilastatin, cimetidine, ciprofloxacin, clonidine, colistin, cosyntropin, cycloserine, daunorubicin, doxorubicin, desmopressin, dihydroergotamine, dobutamine, dopamine, ephedrine, epinephrine, ε-aminocaproic acid, ergometrine, esmolol, famotidine, flecainide, folic acid, flucytosine, furosemide, ganciclovir, gentamicin, glucagon, hydrazaline, imipenem, isoproterenol, ketamine, liothyronine, LHRH, merpatricin, metaraminol, methyldopa, metoclopramide, metoprolol, mexiletine, mitomycin, neomicin, netilmicin, nimodipine, nystatin, octreotide, oxytocin, pamidronate, pentamidine, phentolamine, phenylephrine, procainamide, procaine, propranolol, ritodrine, sotalol, teicoplanin, terbutaline, thiamine, tiludronate, tolazoline, trimethoprim, tromethamine, vancomycin, vasopressin and vinblastine.

Preferred examples of active pharmaceutical ingredients having an NH_2 group as partner in the coupling reaction with HAS are 6-aminopenicillic acid, 7-aminocephalosporin, 7-aminocephalosporanic acid and 7-aminopenicillanic acid.

Specific examples of those active ingredients having a COOH group as partner for the coupling reaction with HAS are:
acetylcysteine, azlocillin, aztreonam, benzylpenicillin, camptothecin, cefamandole, cefazolin, cefepime, cefotaxime, cefotetan, cefoxitin, ceftazidime, ceftriaxone, cephalothin, cilastatin, ciprofloxacin, clavulanic acid, dicloxacillin, ε-aminocaproic acid, floxacillin, folinic acid, furosemide, fusidic acid, imipemem, indomethacin,

ketorolac, liothyronine, melphalan, methyldopa, piperacillin, prostacyclin, prostaglandins, teicoplanin, ticarcillin and vancomycin.

Specific examples of those active ingredients having an aryl-OH group as partner in the coupling reaction with HAS are:
albuterol, allopurinol, apomorphine, ceftriaxone, dobutamine, dopamine, doxycycline, edrophonium, isoproterenol, liothyronine, metaraminol, methyldopa, minocycline, pentazocine, phenylephrine, phentolamine, propofol, rifamycins, ritodrine, teicoplanin, terbutaline, tetracycline and vancomycin.

Specific examples of those active ingredients having an aliphatic OH group as partner in the coupling reaction with HAS are are Taxol and palcitaxel.

The reaction products of the chemical coupling described above can be investigated by known methods, and the coupling efficiency can be established. For example, a UV calibration plot for the relevant low molecular substance can be constructed and used to determine the content of low molecular weight substance in the sample or the proportion of low molecular weight substance in the coupling product. If the low molecular weight substance shows no UV absorption, appropriate colorimetry or electrochemical detection methods can be developed in analogy to known methods. The saccharide content in the conjugate can be detected for example by a glycan-specific staining of the fractionated reaction products. Quantitative glycan determination is also possible. The coupling yield of reactions involving primary amines could also be established by derivatization of the unreacted amines with fluorescamine and determination of the fluorescence.

The improved solubility in water can easily be checked in the case of slightly soluble starting materials by

dissolution tests. In the case of coupling with partially water-soluble active pharmaceutical ingredients, the increased hydrophilicity can be determined by means of an OECD method to measure the logP value. This correlates the retention time of substances in RP-HPLC with the partition coefficient in an n-octanol/water mixture. All HES conjugates of the invention investigated by this method eluted in the hold-up volume of a C18 column, and thus showed no interactions with the C18 material.

The conjugates of the present invention can where appropriate be employed as such or in the form of a pharmaceutical composition for the prophylactic or therapeutic treatment of the human or animal body.

Compositions of this type include a pharmaceutically effective amount of a conjugate of the invention as active ingredient, and a pharmaceutically suitable carrier and, where appropriate, other therapeutic or pharmaceutical ingredients or excipients. Excipients may include for example diluents, buffers, flavorings, binders, surface-active agents, thickeners, lubricants, preservatives (including antioxidants) and substances which serve to make the formulation isotonic with the blood of the intended recipient. A pharmaceutically effective amount is the amount sufficient to display on single or multiple administration a desired beneficial effect during a treatment to alleviate or cure or prevent a pathological condition. A pharmaceutically acceptable carrier is a carrier which is compatible both with the active pharmaceutical ingredient and with the patient's body.

The form of the composition will vary depending on the desired or suitable administration route. Suitable administration routes may be for example oral, parenteral, e.g. subcutaneous, intramuscular, intravenous, intraarterial, intraarticular, intrathecal,

extradural injection or, where appropriate, infusion, intranasal, intratracheal, rectal or topical administration. The pharmaceutical compositions may beneficially be supplied in the form of a dosage unit and be produced by any method well known in the pharmacy sector.

The HAS conjugates of the present invention can also be employed in all other sectors in which other polymer conjugates, e.g. PEG conjugates, have been used. Some specific, non-restrictive examples are the use of an HAS conjugate as immobilized reactant for a reaction in heterogeneous phase or as column material for affinity chromatography. Further possible uses will be plainly evident to the skilled worker with knowledge of the properties disclosed herein of the HAS conjugates of the invention.

The following examples are intended to explain the invention in more detail without, however, restricting it thereto. In particular, analogous reactions can also be carried out with hydroxymethylstarch and hydroxypropylstarch, and similar results can be achieved.

EXAMPLE 1

Selective oxidation of hydroxyethylstarch (HES) with iodine

10 g of HES-130 kD were dissolved in 12 ml of deionized water by heating in a round-bottomed flask. 2 ml of an I_2 solution (0.1N) were added to this solution. A pipette with 2 ml of 1.0N NaOH was connected to the flask via a 2-way connector, and the NaOH solution was added dropwise at about 1 drop every 4 minutes. The solution was decolorized after addition of approximately 0.2 ml of the NaOH solution and, at this time, a second portion of 2 ml of 0.1N iodine solution was added. The reaction was complete after addition of

a total of 14 ml of iodine solution and 2.8 ml of NaOH solution. The reaction mixture was then dialyzed against deionized water.

Lactonization:

The partially desalted solution was subjected to a chromatography on a cation exchange column (Amberlite IR-120, H^+ form) in order to convert the aldonate groups into aldonic acid groups. Subsequently, the water was removed by lyophilization, and thus the lactone form was obtained.

Determination of the degree of oxidation:

1 ml of alkaline copper reagent (3.5 g of Na_2PO_4, 4.0 g of K Na tatrate in 50 ml of H_2O, plus 10 ml of 1N NaOH, 8.0 ml of 10% strength (weight/volume) $CuSO_4$ solution and 0.089 g of K iodate in 10 ml of H_2O, after addition of 18 g of Na sulfate, make up to 100 ml) are pipetted into 1 ml of sample solution in each case under an N_2 atmosphere. The mixture is heated at 100°C for 45 minutes. After cooling, 0.2 ml of 2.5% strength KI solution and 0.15 ml of 2M H_2SO_4 are added. After 5 min, 1 drop of phenol red indicator solution (1% weight/volume) is added, and titration is carried out with 5 mM $Na_2S_2O_3$ solution until the color disappears. The concentration of unreacted aldehyde groups can be calculated from the consumption of titrant.

An approximately quantitative yield was achieved (> 98%). It is possible by this procedure to oxidize hydroxyethylstarches with higher molecular weight (e.g. 130 kD, 250 kD, 400 kD) just like hydroxyethylstarches with lower molecular weight (e.g. 10 kD, 25 kD, 40 kD), in similarly high yields.

EXAMPLE 2

Selective oxidation of HES with Cu^{2+} ions

A solution of 0.24 mmol of HES-130 kD was prepared in 10 ml of deionized water with heating. This solution was heated in a 100 ml round-bottomed flask to a temperature of 70-80°C, and 1.17 mmol of stabilized Cu^{2+} (e.g. Rochelle salt as stabilizer or other stabilizers) and dilute aqueous NaOH solution was added (final concentration 0.1N NaOH). The temperature was then raised to 100°C, and the reaction was allowed to proceed until a reddish color had appeared. The reaction was stopped and the reaction mixture was cooled to 4°C. The reddish precipitate was removed by filtration. The filtrate was dialyzed against deionized water and then converted into the lactone as in Example 1. The oxidation took place quantitatively (yield > 99%). It was also possible by this method to oxidize low molecular weight HES (e.g. HES-10 kD, HES-25 kD, HES-40 kD) and higher molecular weight HES species (e.g. 130 kD, 250 kD, 400 kD).

EXAMPLE 3

Coupling of selectively oxidized hydroxyethylstarch (ox-HES) to alendronate

5 mg of alendronate (a bisphosphonate) and a 3-5-fold molar excess of ox-HES lactone (prepared as described in Example 1 or 2) were dissolved in 4-5 ml of DMSO in a 100 ml round-bottomed flask. The suspension was heated to 70°C and left for 24-36 hours with moderate stirring (magnetic stirrer). The reaction was then stopped and the reaction mixture was cooled to room temperature. Then 20-30 ml of water were added, and this solution was dialyzed against distilled water. Instead of dialysis it is also possible to employ an ultrafiltration with a suitable exclusion limit of the membrane. This makes it possible not only to exchange the solvent but also to concentrate the solution, which

is subsequently lyophilized. The success of the coupling is demonstrated by means of standard analytical methods, e.g. gel permeation chromatography and ninhydrin test for free amino groups. The yield of coupling product was about 85% for the coupling with ox-HES-130 kD and about 80% for coupling with ox-HES-10 kD lactone.

EXAMPLE 4

Coupling of selectively oxidized HES (ox-HES) to amphotericin B

12.0 g of dried ox-HES-130 kD lactone were dissolved in 30 ml of dry DMSO in an N_2 atmosphere. The solution was heated to 70°C, and 52 mg of amphotericin B were added. The reaction was left with exclusion of light under these conditions for 24 h. Successful coupling was demonstrated by gel permeation chromatography with photometric detection at 385 nm (λ_{max} of amphotericin). After completion of the reaction, it was stopped by adding 80 ml of distilled water and intensively dialyzed against water. Lyophilization afforded a pale yellow coupling product. (Yield about 87%).

Under comparable conditions, a yield of about 75% was achieved in the coupling of ox-HES-10 kD lactone with amphotericin B.

EXAMPLE 5

Coupling of ox-HES to ampicillin

1.3 g of dry ox-HES-130 kD lactone were dissolved in 5 ml of dry DMSO in a 100 ml round-bottomed flask. This solution was heated to 45°C, and 11.0 mg of ampicillin (Aldrich # 27.186-1) were added. The reaction took place with moderate stirring for 20 h and was stopped after this time by adding 25 ml of distilled water. The reaction mixture was dialyzed against distilled water and then lyophilized. The success of coupling was

demonstrated by analyzing the product with GPC and determining the free amino groups on the ampicillin using ninhydrin.

EXAMPLE 6

Coupling of ox-HES to neomycin

3×10^{-5} mol of ox-HES-25 kD lactone were dissolved in 5 ml of N-methylpyrrolidone in a 50 ml reaction vessel at 60°C with magnetic stirring. Addition of 10 mg of neomycin in 2 ml of dry DMSO was followed by boiling under reflux for about 10 h. After cooling to room temperature, the reaction was stopped by adding a further 35 ml of water. Most of the solvent was removed by dialysis, and the coupling product was then lyophilized. It was possible to demonstrate coupling product in a yield of about 82% by GPC with UV detection.

EXAMPLE 7

Coupling of ox-HES to mepartricin

10 ml of ethylene glycol were needed to completely dissolve 2.5 g of ox-HES-130 kD lactone and 22 mg of mepartricin (obtainable from Societá Prodotti Antibiotici, Milan, Italy) with heating. The solvent had previously been degassed and dried. The reaction solution was stirred with exclusion of light under an inert gas atmosphere for 36 h, and the reaction was finally stopped by introducing 40 ml of ice-cold water. The ethylene glycol was removed by ultrafiltration (10 kD membrane), and subsequent lyophilization afforded 2.1 g of pale yellowish powder. Further purification took place by RP-HPLC on a C18 column with UV/VIS detection.

EXAMPLE 8

Coupling of ox-HES to nystatin

2.5 g of dry ox-HES-130 kD lactone were dissolved in 10 ml of dry DMSO in a 100 ml round-bottomed flask. Addition of 9.5 mg of nystatin was followed by heating to 60°C and stirring in the dark under an inert gas atmosphere. The reaction took place with moderate stirring for 48 h and was stopped after this time by adding 50 ml of distilled water. The reaction mixture was dialyzed against distilled water and then lyophilized. Successful coupling was demonstrable by RP-HLPC (C18 column) and detection at 325 nm. The yield estimated from the absorption of the product peak was about 67%.

EXAMPLE 9

Coupling of ox-HES to mitomycin C

2.5 g of ox-HES-130 kD lactone and 20 g of mitomycin (Fluka # 69824) were dissolved in 10 ml of a 9:1 DMSO:MeOH mixture at 60°C. The reaction solution was kept under reflux for 24 h and then 40 ml of water were added to stop the reaction. This solution was dialyzed against deionized water overnight and then subjected to a freeze drying. Coupling was demonstrated by RP-HPLC and detection at 320 nm. The expected coupling product resulted in a yield of 82%.

EXAMPLE 10

Coupling of ox-HES to daunorubicin

1.3 g of ox-HES-130 kD lactone were dissolved in 10 ml of N-methylpyrrolidone with stirring at 70°C. 17 mg of daunorubicin (Fluka #30450), dissolved in 3 ml of DMF, were added dropwise thereto. The reaction mixture was stirred under these conditions for 20 h, cooled to room temperature and finally shaken with 40 ml of distilled water. Most of the solvent was removed by dialysis

against water, followed by freeze drying. The coupled daunorubicin was demonstrated by RP-HPLC and UV-VIS detection.

EXAMPLE 11

Coupling of ox-HES to 7-aminocephalosporin

3.0 g of ox-HES-130 kD lactone and 20 mg of 7-aminocephalosporin (Fluka #07300) were dissolved in 5 ml of dry DMSO in a 100 ml round-bottomed flask with magnetic stirring. The temperature was raised to 50°C and maintained for 15 h. After this time, the reaction mixture was cooled to 25°C and diluted by adding 5 ml of distilled water. DMSO and unreacted 7-aminocephalosporin were removed by dialysis against distilled water. The solution was then lyophilized and the product was analyzed by TLC and GPC.

EXAMPLE 12

Coupling of ox-HES to 6-aminopenicillic acid

The reaction described in Example 11 was also carried out with 16 mg of 6-aminopenicillic acid instead of 7-aminocephalosporin under the same conditions, and the reaction product was worked up and analyzed under the same conditions.

EXAMPLE 13

Coupling of ox-HES to LHRH

1.0 g of dried ox-HES-130 kD lactone was incubated with 5 mg of LHRH (luteinizing hormone-releasing hormone) (Bachem, Switzerland) in 10 ml of dry DMSO. The reaction proceeded while stirring at 45°C for 15 h and was stopped by adding 40 ml of water. Hesylated LHRH was obtained by lyophilization after it had been extensively dialyzed against water in order to remove most of the DMSO and unreacted peptide. The resulting product was analyzed by GPC (Superose 12, Amersham-

Pharmacia, Sweden) and UV detection at 280 nm. A stoichiometry of approximately 1:1 for the coupling product emerged from the quantification of the peptide on the basis of the Trp absorption and the quantification of the polysaccharide content by phenol/sulfuric acid coloring.

EXAMPLE 14

Coupling of ox-HES to camptothecin

20 mg of camptothecin were dissolved in 5 ml of dry DMSO at 50°C in a round-bottomed flask. 36 mg of 1.4-diaminobutane in 2 ml of dry DMSO were added dropwise to this solution. The reaction mixture was left to stir gently under these conditions for 24 h. The conjugation product was purified by flash chromatography. The yield was about 83%.

For the coupling reaction of the modified camptothecin with ox-HES-130 kD, the complete reaction mixture was dissolved after purification together with 3.6 g of the polysaccharide lactone in 8 ml of dry DMSO by stirring and heating at 50°C. The progress of the reaction was followed by RP-HPLC of samples from the reaction mixture. After 20 h at 50°C, no further product formation was observable, and the reaction was stopped by adding 50 ml of distilled water. After dialysis against water, the coupling product was freeze dried. Analysis took place by GPC and staining of the free amino group in the modified, unreacted camptothecin with ninhydrin on a TLC plate.

EXAMPLE 15

Coupling of ox-HES to prostacyclin

a) Amino functionalization

352 mg of prostacyclin (Sigma-Aldrich) were dissolved in 5 ml of dry DMF with 2% methylene chloride (V/V) at

0°C. 1.3 g of dicyclohexylcarbodiimde (DCC) in 5 ml of dry DMF were added thereto. Reaction was allowed to take place while stirring gently for 30 minutes. Then a 5-fold molar excess (based on prostacyclin) of 1.5-diaminoethyl ether was added, and the solution was slowly warmed to room temperature. The amino-functionalized coupling product was purified by flash chromatography on a silica phase.

b) Hesylation

220 mg of the purified coupling product from a) were dissolved in 8 ml of glycol at room temperature. 4.0 g of ox-HES-130 kD lactone, dissolved in 10 ml of glycol, were admixed with stirring and heated to 45°C. After a reaction time of 8 h, the mixture was cooled in an ice bath and dialyzed intensively against water. The clear solution was investigated by RP-HPLC on a C18 column. It was possible to calculate the coupling efficiency from the ratio of the areas in the hold-up volume of the column (coupling product) and the initial substance. The yield was 53%.

EXAMPLE 16

Coupling of HES to alendronate

A ten-fold molar excess of HES-25 kD was added to a solution of 2.25 mg of alendronate in 4 ml of phosphate buffer (0.1M, pH 7.5) in a 100 ml round-bottomed flask. The reaction mixture was shaken in order to dissolve the polysaccharide completely, and then a thirty-fold molar excess of $NaBH_3CN$ was added. The reaction proceeded at room temperature for 48 h, the production of a coupling product being detected in an aliquot by reaction with fluorescamine, which yields a fluorescent product with free amino groups.

EXAMPLE 17

Coupling of HES to amoxillin

4.0 ml of 0.1N Na phosphate buffer (pH 7.5) were introduced into a two-neck flask, and 1.5 g of HES-40 kD were dissolved therein by heating to 60°C. After cooling to 25°C, 7.0 mg of amoxillin (Fluka #10039) were added with magnetic stirring. A solution of $NaBH_3CN$ corresponding to a thirty-fold molar excess was prepared in 2 ml of the same Na phosphate buffer in a separate vessel. The cyanoborohydride solution was slowly added dropwise, using a dropping funnel, to the first solution over a period of 30 minutes. The reaction mixture was stirred for a further 24-36 h and then the pH was adjusted to 4 with 0.1N HCl to stop the reaction. The solution was desalted by dialysis and lyophilized. Demonstration of the coupling product took place by GPC and UV photometer.

EXAMPLE 18

Coupling of HES to cefaclor

4 ml of 0.1N Na phosphate buffer (pH 7.0) were used to dissolve 110 mg of $NaBH_3CN$ in a 100 ml round-bottomed flask. 6.0×10^{-5} mol of HES-130 kD and 2.0×10^{-5} mol of cefaclor (Fluka #22125) were added while stirring. The reaction temperature was kept at 25°C, and the reaction mixture was stirred moderately for 24 h. The solution was then acidified to pH 4.0 and stirred for a further 30 minutes. Desalting and concentration were carried out by ultrafiltration (10 kD membrane). The coupling product was demonstrated by HP-GPC at 265 nm.

EXAMPLE 19

Coupling of HES to doxorubicin

6.0 mg of doxorubicin (Fluka #45584) were suspended in 4 ml of 0.1N Na phosphate buffer (pH 7.5) in the presence of a three-fold molar excess of HES-130 kD at

room temperature. The reaction mixture was vigorously stirred for 30 minutes, and 3 ml of a 0.8M $NaBH_3CN$ solution was slowly added. The reaction was kept at room temperature with stirring for 48 h. A 10 kD membrane was then used for diafiltration in order to remove salts and unreacted doxorubicin. The diafiltered solution was lyophilized and the coupling product was investigated by GPC and RP-HPLC.

EXAMPLE 20

Coupling of HES to vasopressin

1.25 g of HES-130 kD were dissolved in 5 ml of 0.1M Na phosphate buffer, pH 8.0, with heating and gentle stirring in a round-bottomed flask equipped with a dropping funnel. 5 mg of vasopressin (Bachem, Switzerland) were added this solution. 30 mg of $NaBH_3CN$ were dissolved in 2 ml of 0.1M phosphate buffer (pH 7.5) and slowly added dropwise through the dropping funnel to the reaction mixture. The reaction was left to stand at 25°C for about 24 h. To terminate the reaction, the pH was lowered to 4.0 by adding 0.1N HCl. After extensive dialysis against water, the hesylated product was freeze dried. Analysis took place by GPC as described above and UV detection at 220 nm.

EXAMPLE 21

Coupling of ox-HES 70 kD to neomycin

1.01 mg of neomycin (sulfate salt) and 126.21 mg of oxHES 70 kD were dissolved in 2 ml of DMSO in a two-neck flask under an argon atmosphere and, after addition of 0.81 mg of DMAP, heated at 70°C for 24 h. The reaction was then stopped by adding acetone, whereupon the coupling product precipitated. The solid was dissolved in water and purified by dialysis against water for 48 h. Freeze drying resulted in 80 mg of white coupling product (63%).

EXAMPLE 22

Alternative method for coupling of ox-HES 70 kD to neomycin

Coupling of neomycin to ox-HES 70 kD can likewise be carried out successfully at room temperature in DMSO with addition of EDC as activator. For this purpose, 16.97 mg of neomycin (sulfate salt), 348 mg of ox-HES 70 kD and 2.28 mg of DMAP were dissolved in 1 ml of DMSO. After addition of 3.83 mg of DCC (1 equivalent), the solution was stirred for 2 h and the addition of one equivalent of DCC was repeated. This process was repeated until 10 equivalents of DCC had been added to the reaction solution. The reaction time totaled 24 h. After addition of 20 ml of acetone to the solution, the coupling product precipitated. The solid was dissolved in water and purified by dialysis against water for 48 h. Freeze drying resulted in 280 mg of white coupling product (80%).

EXAMPLE 23

Coupling of ox-HES 70 kD to daunorubicin

0.5 mg of daunorubicin hydrochloride, 829.2 mg of ox-HES 70 kD and 0.108 mg of DMAP were dissolved in 2 ml of DMSO under an argon atmosphere in a two-neck flask and heated at 70°C for 24 h. Then acetone (20 ml) was added thereto, whereupon the coupling product precipitated. The solution was centrifuged and the precipitate was washed with acetone and centrifuged several times. A pale pink-colored solid was obtained and was dissolved in water and dialyzed against water. Freeze drying results in 656 mg (80%) of a pale pink-colored solid. The purity of the coupled daunorubicin was checked by RP-HPLC.

EXAMPLE 24

Coupling of ox-HES 130 kD to 7-aminocephalosporanic acid

383 mg of ox-HES-130 kD and 1.22 mg of 7-aminocephalosporanic acid (Fluka #07300) were dissolved in 2 ml of dry DMSO in a 100 ml round-bottomed flask with magnetic stirring. The temperature was raised to 70°C and maintained for 24 h. After this time, the mixture was cooled to 25°C, and the reaction product was precipitated by adding 20 ml of acetone. The solid was washed with 20 ml of acetone and dissolved in 20 ml of distilled water. Further purification of the coupling product took place by dialysis against distilled water. The solution was then lyophilized and the product was analyzed by TLC and GPC. 270 mg of coupling product (70%) were obtained in the form of a white solid.

EXAMPLE 25

Coupling of ox-HES 70 kD to 6-aminopenicillanic acid

The reaction described in Example d was also carried out with 1.57 mg of 6-aminopenicillanic acid instead of 7-aminocephalosporanic acid and 135.54 mg of ox-HES 70 kD under the same conditions. The reaction product was worked up and analyzed under the same conditions. After purification, 88 mg of coupling product (65%) were obtained as white solid.

EXAMPLE 26

Coupling of HES 40 kD to amoxicillin

4.0 ml of 0.1N Na phosphate buffer (pH 7.5) were introduced into a two-neck flask, and 1.5 of HES-40 kD were dissolved therein by heating to 60°C. After cooling to 25°C, 7.0 mg of amoxicillin (Fluka #10039) were added with magnetic stirring. A solution of $NaBH_3CN$ corresponding to a thirty-fold molar excess was prepared in 2 ml of the same Na phosphate buffer in a

separate vessel. The cyanoborohydride solution was slowly added dropwise with the aid of a dropping funnel to the first solution over a period of 30 minutes. The reaction mixture was stirred for a further 24-36 h, and then the pH was adjusted to 4 with 0.1N HCl to stop the reaction. The solution was desalted by dialysis and lyophilized. Demonstration of the coupling product took place by GPC and UV photometer.

EXAMPLE 27

Coupling of ox-HES 70 kD to amoxicillin

173 mg of ox-HES 70 kD and 0.85 g of amoxicillin were dissolved in 2 ml of dry DMSO in a 100 ml round-bottomed flask with magnetic stirring. The temperature was raised to 70°C and maintained for 24 h. After this time, the mixture was cooled to 25°C, and the reaction product was precipitated by adding 20 ml of actone. The solid was washed with 20 ml of acetone and dissolved in 20 ml of distilled water. Further purification of the coupling product took place by dialysis against distilled water. The solution was then lyophilized, and the product was analyzed by TLC and GPC. 151 mg of coupling product (87%) are obtained in the form of a white solid.

EXAMPLE 28

Coupling of ox-HES 70 kD to cefadroxil

610 mg of ox-HES 70 kD and 2.965 mg of cefadroxil were dissolved in 2 ml of dry DMSO in a 100 ml round-bottomed flask with magnetic stirring. The temperature was raised to 70°C and maintained for 24 h. After this time, the mixture was cooled to 25°C, and the reaction product was precipitated by adding 20 ml of actone. The solid was washed with 20 ml of acetone and dissolved in 20 ml of distilled water. Further purification of the coupling product took place by dialysis against distilled water. The solution was then lyophilized, and

the product was analyzed by TLC and GPC. 490 mg of coupling product (87%) are obtained in the form of a white solid.

EXAMPLE 29

Coupling of ox-HES 70 kD to glucagon

Glucagon (66×10^{-9} mol, 0.23 mg), oxHES 70 kD (6.6×10^{-6} mol, 123 mg) are dissolved in 1 ml of DMSO in a round-bottomed flask. At intervals of 1 h, DDC is added in 8 portions at 1 h intervals until a total of 23.08 mg have been added to the reaction solution. After a reaction time of 24 h, the reaction is stopped by adding 15 ml of water. The coupling product purified by dialysis against water. Freeze drying results in 79 mg of white coupling product (65%).